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MICROBIAL INTERACTIONS WITH MYCOTOXIGENIC FUNGI AND MYCOTOXINS

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Mycotoxins such as aflatoxins, fumonisins, trichothecenes, and ochratoxins are contaminants of many agronomic crops worldwide, and cause both economic losses and health effects. The potential of antagonistic microorganisms to be developed into biological control agents has been investigated in several crop systems, as alternatives to chemical fungicides for control of mycotoxigenic fungi. Laboratory and greenhouse studies have identified a number of bacterial, yeast, and filamentous fungal isolates that reduce crop contamination of mycotoxigenic fungi, although investigations of field efficacy have been limited. These studies demonstrate that the diversity of ecological interactions between mycotoxigenic fungi and other resident microorganisms may provide tools for development of biocontrol methods to reduce mycotoxin contamination.

Keywords: *Aspergillus*, *Fusarium*, *Penicillium*, mycotoxins, biocontrol, microbe-microbe interactions, microbial ecology

Introduction

Severalgenera and species of filamentous fungi produce polyketide-derived mycotoxins that have significant agricultural, epidemiological and economic impact. *Aspergillus*, *Fusarium*, and *Penicillium* species are responsible for the majority of agricultural mycotoxin contamination. These fungi are common components of the microbial flora associated with many agronomic crops,

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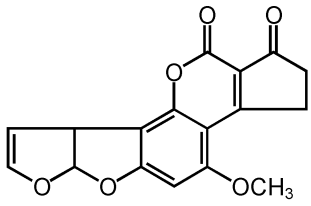
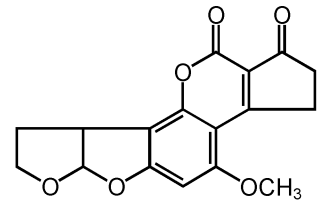
including maize, peanuts, tree nuts, grapes, coffee, cotton, wheat, barley, and other cereal grains. Depending on the host crop and the fungal species, mycotoxigenic fungi may cause plant disease, such as *Aspergillus* fruit rot of grapes, maize ear rots caused by *Aspergillus* and *Fusarium* species, and *Fusarium* head blight and seedling blight diseases on cereal crops. Thus, these phytopathogenic interactions result in further crop loss and economic impact. In contrast, other host-fungal interactions do not cause disease symptoms, such as epiphytic growth of mycotoxigenic *Aspergilli* on tree nuts, or asymptomatic, endophytic growth of *Fusarium* in maize leaves.

The major agriculturally important mycotoxins, shown in Figure 1, include aflatoxins, fumonisins, the trichothecene mycotoxin deoxynivalenol, and ochratoxins, each of which is produced by several fungal species. *Aspergillus flavus* and *A. parasiticus* are the major producers of aflatoxins; *Fusarium verticillioides* and *F. proliferatum* produce fumonisins; several *Fusarium* species, including *F. graminearum* and *F. culmorum*, produce trichothecenes; and several *Aspergillus* and *Penicillium* species produce ochratoxins. The impact of these classes of mycotoxins on human and animal health has been extensively studied (Council for Agricultural Science and Technology, 2003). Thus, these mycotoxins are of considerable food safety concern, which has led to regulatory action to limit contamination of agricultural commodities used for food and feed.

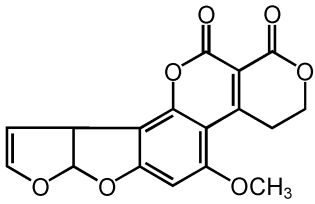
Effective control of mycotoxigenic fungi traditionally has been through the use of chemical fungicides, modifications in cultural practices, and development of resistant cultivars. In addition, methods of postharvest sorting of contaminated products, such as maize, wheat, peanuts and tree nuts, have been developed to reduce mycotoxin content in those commodities (Schatzki and Haddon, 2002; Campbell et al., 2003; Pearson et al., 2004; Delwiche et al., 2005). Biological control methods also have been investigated for controlling mycotoxigenic fungi. One example of effective biocontrol has been the use of nonaflatoxigenic *A. flavus* strains to competitively exclude aflatoxin-producing *Aspergillus* on cotton and peanut, reviewed elsewhere (Dorner, 2004; Cotty, 2006). Several other bacterial and fungal antagonists of mycotoxigenic fungi have been investigated, with the goal of developing other biocontrol agents for mycotoxin reduction. The purpose



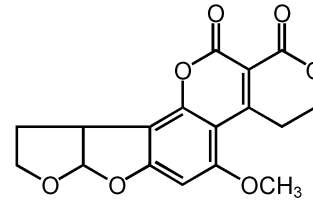
Aflatoxin B_1



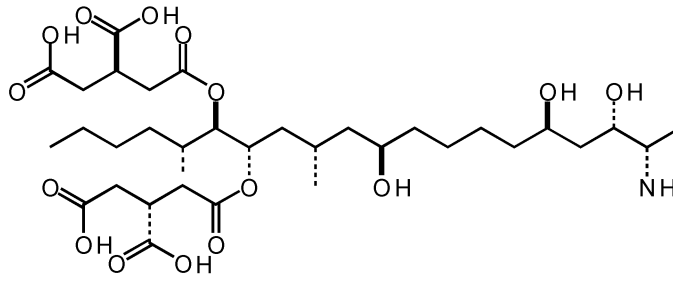
Aflatoxin B_2



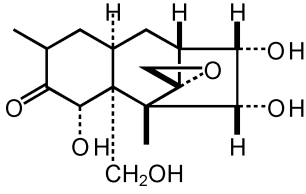
Aflatoxin G_1



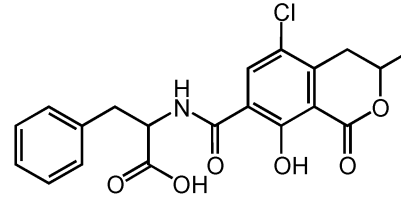
Aflatoxin G_2



Fumonisin B1



Deoxynivalenol



Ochratoxin A

FIGURE 1 Chemical structures of mycotoxins addressed in this review.

of this review is to summarize recent studies of such interactions, which provide insights into potential ecological mechanisms for biocontrol using naturally occurring microorganisms. Also, investigations into microbial interactions with mycotoxigenic fungi

may shed light on the ecological value of mycotoxins to the fungi that produce them.

Bacterial and Fungal Interactions with Aflatoxin-Producing *Aspergillus*

Based on human toxicity and widespread infection of crops, aflatoxin is easily considered the most agriculturally important mycotoxin. The presence of this toxin in agronomic commodities leads to loss of revenue in food and feed production. For example, in 1998, corn producers in Arkansas, Mississippi, Texas, and Louisiana experienced severe aflatoxin contamination, resulting in a commodity rejection rate of over 50% at river terminals (Wrather and Sweets, 2008). Aflatoxins are furanocoumarin derivatives (Figure 1) produced by several species of *Aspergillus* and at least two species of *Penicillium*. Of these, *A. flavus* and *A. parasiticus* are the major producers of aflatoxin. One difference between these species is that *A. flavus* typically produces aflatoxins B_1 and B_2, and *A. parasiticus* typically produces aflatoxins B_1, B_2, G_1, and G_2. Currently, the ecological importance of the different forms of aflatoxin, and of aflatoxin production in general, is not fully understood.

Several species of bacteria, fungi, and yeasts have been studied recently with regard to their interactions with *A. flavus* and *A. parasiticus*, with the goal of developing potential agents for biological control. In laboratory studies, Taylor and Draughon (Taylor and Draughon, 2001) examined the antifungal activity of a bacterial micropredator, *Nannocystis exedens*, against aflatoxigenic fungi. The bacterium is a strain of myxobacteria, a group of Gram-negative, soilborne bacteria characterized by swarming motility and multicellular behavior. They found that *N. exedens* produced antifungal metabolites in culture that inhibited *A. flavus* and *A. parasiticus* growth and sporulation. In addition, this bacterium was observed to aggregate upon and lyse *A. flavus* and *A. parasiticus* conidia, germ tubes, and mycelia, presumably by the production of cell wall–lytic extracellular enzymes. These dual activities of antibiosis and parasitism of aflatoxigenic *Aspergilli* may be the means by which aflatoxin- and other mycotoxin-producing fungi are naturally suppressed in soil; outside the lab, *N. exedens* and other myxobacteria play an important ecological role as predators

to control populations of fungal and bacterial pathogens. It is possible, therefore, that this group of bacteria may be useful in developing targeted strategies for controlling fungi in agricultural systems.

Another example of cultural experimentation to identify microbial antagonists of aflatoxigenic fungi is the characterization of the inhibitory activity of two *Lactobacillus* species against *A. flavus* (Bueno et al., 2006). Strains of *L. casei*, isolated from human feces, and *L. rhamnosus*, isolated from yogurt, both reduced growth of *A. flavus* strains in liquid co-culture, but not in agar diffusion assays. These results were attributed to the acidification of the liquid medium to a level inhibitory to fungal growth, but since lactic acid bacteria generally produce several types of antifungal compounds, such as organic acids, hydrogen peroxide, and low molecular weight secondary metabolites, inhibition of *A. flavus* may be the result of a combination of factors.

Several other in vitro studies have shown bacterial inhibition of aflatoxin production in *A. flavus* and *A. parasiticus* without concurrent inhibition of fungal growth. Coallier-Ascah and Idziak (Coallier-Ascah and Idziak, 1985) demonstrated that when *A. flavus* was grown in a preexisting culture of a strain of *Streptococcus lactis*, aflatoxin production was inhibited. This effect was shown not to be the result of bacterial acidification of the medium (to pH 4.3) or bacterial utilization of glucose prior to fungal inoculation, but to the production of an extracellular inhibitor. In addition to inhibiting new aflatoxin production, *S. lactis* was also shown to degrade and thereby detoxify preformed aflatoxins B_1 and G_1 in spent *A. flavus* culture. Interestingly, *A. flavus* in turn inhibited *S. lactis* growth and caused morphological changes in bacterial cells, effects attributed to fungal extracellular compounds other than aflatoxins.

Specific compounds that have been studied that affect aflatoxin production include aflastatin A and cyclo(L-leucyl-L-prolyl). Aflastatin A is a large polyketide secondary metabolite isolated from a *Streptomyces* sp. strain (Ono et al., 1997). This compound was isolated originally due to its inhibition of aflatoxin production by *A. parasiticus*, and was found additionally to inhibit growth of other fungi and Gram-positive bacteria, as well as repress adenocarcinoma tumor growth in mice. Aflastatin A inhibited aflatoxin production at 0.5 μg/ml, and antimicrobial activity

occurred at MIC levels of less than 1 μg/ml. Cyclo(L-leucyl-L-prolyl) was isolated from the supernatant of a strain of *Achromobacter xylosoxidans* that inhibited norsolorinic acid accumulation in a mutant strain of *A. parasiticus* (Yan et al., 2004). This compound showed complete inhibition of aflatoxin production at 3.5 mg/ml, which corresponded to repressed expression of aflatoxin biosynthesis genes. These studies showing microbial suppression of aflatoxigenesis are useful starting points to determine whether such phenomena occur in natural ecosystems, and whether they can be exploited to limit mycotoxin contamination in agricultural environments.

Other researchers have looked for microorganisms to control aflatoxigenic fungi by specifically screening isolates found in environments native to *A. flavus* and *A. parasiticus*, including plant parts and soil microenvironments. In cotton, bacteria were isolated from field soil, cotton seeds, stems, leaves, flowers, and interior and exterior tissues of cotton bolls (Misaghi et al., 1995). Bacteria were screened for their ability to inhibit fungal colonization of cotton seeds in liquid co-culture with *A. flavus*. The strains that showed antagonistic activity were isolated from immature bolls, mature bolls, or seed from mature bolls, environments susceptible to aflatoxin contamination. Field trials on cotton to test biocontrol potential were conducted by coinoculating mechanically damaged, immature bolls with *A. flavus* spores and bacterial suspensions. One bacterial isolate, identified as *Pseudomonas* (now *Burkholderia*) *cepacia*, reduced the severity of boll damage caused by *A. flavus* infection by 65% over four trials. However, *B. cepacia* is currently of limited use as a biocontrol agent in the United States, due to its association with opportunistic human infections (Parke, 2000). Currently, strain differentiation using multilocus sequence typing (Vandamme and Mahenthiralingam, 2003; Baldwin et al., 2005) has demonstrated that environmental and clinical *B. cepacia* strains cannot be distinguished.

Inhibition of *A. flavus* infection by resident bacterial isolates has also been studied in peanut environments. Anjaiah and colleagues (Anjaiah, 2006) isolated several strains of *Pseudomonas* and *Bacillus* from soils associated with peanut pods, and showed inhibition of *A. flavus* growth in dual-culture laboratory assays, presumably via antibiosis. Two of these isolates, one *Pseudomonas* sp. and one *Bacillus* sp., were tested for reduction of *A. flavus*

populations on pods, pod infection, and seed infection in greenhouse pot assays and in field trials. In greenhouse and field assays, respectively, the *Bacillus* isolate reduced *A. flavus* populations by 75% and 81%, pod infection incidence by 64% and 59%, and seed infection incidence by 56% and 50%. Likewise, the *Pseudomonas* isolate reduced *A. flavus* populations by 77% and 75%, pod infection incidence by 77% and 59%, and seed infection incidence by 44% and 65% under greenhouse and field conditions, respectively.

In addition to bacterial residents of the peanut rhizosphere and seed pods, *A. flavus* also encounters other fungi such as *Trichoderma* (Anjaiah, et al., 2006). Naturally occurring isolates of *T. longibrachiatum*, *T. viride*, and *T. harzianum* showed inhibition of *A. flavus* geocarposphere populations in greenhouse and field studies of 37% to 95% and 62% to 75%, respectively. *A. flavus* infection levels of developing pods and seeds were reduced in greenhouse experiments by 53% to 74% and 13% to 52%, respectively. In field experiments, *Trichoderma* isolates inhibited pod and seed infection of *A. flavus* by 44% to 63% and 38% to 68%, respectively. For both bacterial and fungal experiments, reductions in *A. flavus* populations and reduction of infection on developing pods and seeds would not necessarily be adequate for effective biological control of *A. flavus* on peanut. However, this study does reveal a microbial community in the peanut soil environment containing resident antagonistic bacteria and fungi, which may be a source for future development into useful biocontrol strategies to reduce resident populations of undesirable fungi.

Several strains of *Bacillus* isolated from the maize environment were investigated recently for their interactions with *A. flavus* and *A. parasiticus*, and to determine the effects of water activity (a_w) on these interactions (Bluma and Etcheverry, 2006). Initial screens to demonstrate in vitro antagonistic phenotypes showed that contact inhibition was a major mechanism of activity of these *Bacillus* strains against *A. flavus* and *A. parasiticus*. Using sterilized maize kernels as a growth substrate, these *Bacillus* strains reduced fungal populations by 30% or less at a_w of 0.982, and were ineffective at a_w 0.955. In maize extract agar and on maize kernels at a_w 0.982, aflatoxin production by both *A. flavus* and *A. parasiticus* was repressed by the *Bacillus* isolates by up to

80%. At lower a_w levels on maize kernels, none of the *Bacillus* strains significantly affected aflatoxin production. Since water activity is an important component affecting growth and aflatoxin production of *A. flavus* and *A. parasiticus*, both in vitro and under field conditions (Widstrom, 1996; Payne, 1998), it is essential that tests of antagonistic behavior of potential biocontrol agents take into account the environmental conditions relevant for control of fungal growth and/or aflatoxin production.

Our laboratory has recently performed studies surveying bacterial populations in the maize ecosystem for antagonistic activity against *A. flavus* (Palumbo et al., 2007). A variety of bacterial genera isolated from maize field soil and rhizosphere samples showed antifungal activity against *A. flavus* in agar and liquid co-culture assays. While *Bacillus* and *Pseudomonas* spp. were common in soil samples, rhizosphere samples yielded greater diversity of antagonistic bacteria, including strains of *Burkholderia*, *Pseudomonas*, *Stenotrophomonas*, *Variovorax*, *Agrobacterium*, *Bacillus*, and *Achromobacter*, as well as a number of plant-associated enteric and coryneform bacteria. In rhizosphere samples, the most frequently isolated antagonists were *Burkholderia* spp. Liquid co-culture assays using potato dextrose broth and maize kernel extract medium demonstrated that *Pseudomonas* and *Burkholderia* isolates were the most consistently inhibitory to *A. flavus* growth in both media. In contrast, *Bacillus* strains showed much lower activity in potato dextrose broth than in maize kernel extract medium, suggesting nutritional regulation of antifungal activity. Several of these isolates produced diffusible antifungal metabolites, as well as extracellular chitinase or β-glucanase activities in culture, suggesting that multiple mechanisms of antagonism may occur in situ. Current work in our laboratory is focused on determining the contribution of each of these factors in bacterial interactions with *A. flavus*. The discrepancy in antifungal activity between *Bacillus* isolates from maize and those described from peanut (Anjaiah et al., 2006) suggests major differences in the microbial activity of bacterial genera in each ecosystem. Therefore, the successful development of biocontrol strategies using resident bacteria may require that each be tailored specifically to individual crop systems and environmental habitats.

Also in maize, Wicklow and colleagues (Wicklow et al., 2005) recently reported the isolation of antifungal metabolites from

Acremonium zeae, a fungal endophyte of maize that has been shown to limit *A. flavus* colonization and aflatoxin contamination of maize (Wicklow et al., 1988). These metabolites were determined to be pyrrocidines A and B, which were shown to be produced by a number of *A. zeae* isolates. In addition to inhibiting *A. flavus* growth, these compounds also inhibit growth of *F. verticillioides*. As *F. verticillioides* and *A. zeae* are both endophytes of maize, interactions between the two may offer interesting insights into competitive traits that function in their endophytic lifestyles.

Several yeast species also have been shown to inhibit *A. flavus* and *A. parasiticus* growth and aflatoxin production in vitro. Six isolates of *Kluyveromyces* spp. were shown to inhibit *Aspergillus* spore germination, germ tube elongation, and hyphal growth rates on maize meal extract media at different a_w levels (La Penna and Etcheverry, 2006). At higher a_w (0.994 and 0.982), all *Kluyveromyces* isolates completely inhibited aflatoxin production in the tested *Aspergillus* strains. Surprisingly, at lower a_w (0.955 and 0.937), certain combinations of *Kluyveromyces* and *Aspergillus* resulted in increased aflatoxin levels relative to control levels. Use of these yeasts as biocontrol agents would be potentially detrimental under drought stress, since aflatoxin production is associated with drought stress in maize (Payne, 1998).

Tree nut environments have also been explored for antagonistic bacteria and yeasts with potential as biocontrol agents. In one study, bacterial populations from almond orchards were surveyed throughout the growing season for antagonistic activity against *A. flavus* (Palumbo et al., 2006). Early in the growing season, antagonists isolated from flowers were almost exclusively *Pseudomonas* spp. A greater diversity of antagonistic bacterial genera was recovered from immature and mature fruits, predominated by *Bacillus* spp. as well as *Pseudomonas*, *Ralstonia*, *Burkholderia*, and *Delftia* spp. In vitro assays indicated that several of these isolates produced diffusible antifungal compounds, as well as extracellular chitinase and β-glucanase activities. In liquid co-culture with *A. flavus*, many of these strains reduced fungal growth to undetectable levels, in both yeast extract sucrose broth and in almond kernel extract medium. As with bacteria isolated from maize soils (Palumbo et al., 2007), the variety of antifungal metabolites and enzyme activities may be ecologically important traits, which may be valuable in development of biocontrol agents

in these systems. In addition, yeasts isolated from almond, pistachio, and walnut trees (Hua et al., 1999) showed inhibition of aflatoxin production by *A. flavus*. These include strains of *Pichia anomala*, *Rhodotorula mucilaginosa*, *Candida krusei*, *C. oleophila*, *C. guilliermondii*, and *Cryptococcus laurentii*. It might be useful to investigate whether combinations of bacteria and yeasts in tree nut environments could further reduce fungal contamination and aflatoxin production in these crops.

Bacterial Interactions with Fumonisin-Producing *Fusarium*

Fumonisins are another group of mycotoxin contaminants found in food and feed products. Fumonisin B1 in particular is of international, agroeconomic, and food safety concern. For example, high doses of fumonisin B1-infested corn feed have been shown to cause pulmonary edema in swine, while lower doses lead to hepatic disease (Haschek et al., 1992). These mycotoxins are produced predominantly by toxigenic strains of *Fusarium verticillioides* (Sacc.) Nirenberg (teleomorph *Gibberella moniliformis* Wineland). The fungus commonly proliferates in maize, causing stalk and ear rot diseases, in addition to mycotoxin contamination.

A number of reports in recent years have focused on interactions between *F. verticillioides* and potential bacterial antagonists from maize environments. Detailed studies of screening criteria to select bacterial antagonists of *F. verticillioides* for use as biocontrol agents were performed by Cavaglieri and colleagues (2004a, 2004b). They determined correlations of niche overlap index (NOI), index of dominance, antibiosis, inhibition of fumonisin production, and effects on fungal growth rate and lag phase between combinations of 11 bacterial strains and 13 *F. verticillioides* strains isolated from the maize rhizosphere (Cavaglieri et al., 2004a). NOI is a measure of competitiveness related to nutrient source utilization common between organisms (Wilson and Lindow, 1994). Higher NOI values, therefore, indicate greater potential for nutrient competition. Of these screening criteria, the only factors that were not correlated were NOI and antibiosis, indicating that the combination of these factors would theoretically provide greater efficacy of a biocontrol agent. In greenhouse experiments, one of these bacterial strains, an isolate of *Azotobacter armeniacus* (subsequently identified as *Microbacterium oleovorans*

[Cavaglieri, Orlando, and Etcheverry, 2005]), showed positive NOI and antibiosis phenotypes and significantly controlled *F. verticillioides* relative to maize root colonization in native soil. In a subsequent study (Cavaglieri et al., 2004b), these 11 bacterial strains were tested in vitro for control of *F. verticillioides* at three different a_w levels: a_w 0.982, a_w 0.955, and a_w 0.937. All bacterial strains inhibited *F. verticillioides* growth on maize meal extract agar by a combination of antibiosis, lag phase extension, and/or growth rate reduction. In addition, all strains of *P. solanacearum* and *B. subtilis* and two strains of *Az. armeniacus* (*M. oleovorans*) significantly inhibited fumonisin B1 production at a_w 0.982 and a_w 0.955.

Further studies focused on the effectiveness of *Az. armeniacus* (*M. oleovorans*), *Ar. globiformis* (*Enterobacter cloacae*), and *Bacillus subtilis* in reducing *F. verticillioides* colonization of maize (Cavaglieri, Andrés, et al., 2005; Cavaglieri, Orlando, Rodríguez, et al., 2005). Using maize field soil in greenhouse assays, *F. verticillioides* rhizoplane and endorhizosphere populations were successfully inhibited when seeds were treated with single strains of *Az. armeniacus* (*M. oleovorans*) and *Ar. globiformis* (*E. cloacae*), as well as a dual treatment of each bacterium. The extent of fungal inhibition varied with bacterial inoculum levels. Depending on the bacterial strain, the highest inoculum levels were not necessarily the most effective at reducing *F. verticillioides*, pointing to the microbial balance that must be maintained in order for some biocontrol mechanisms to be effective.

Together, the studies by Cavaglieri and colleagues demonstrate the utility of multiple screening criteria for the selection of effective bacterial agents with great potential for biocontrol of *F. verticillioides*, and thus fumonisin contamination, of maize. Furthermore, the combined influence of mixed maize rhizosphere bacterial strains on *F. verticillioides* growth was examined (Cavaglieri, Orlando, and Etcheverry, 2005). Mixtures of *E. cloacae* and *M. oleovorans*, or *P. solanacearum* and *B. subtilis* (described in previous studies), showed differential effects on *F. verticillioides* on maize meal extract agar relative to antibiosis, growth rate inhibition, and fumonisin B1 production at different a_w levels. The combination of *E. cloacae* and *M. oleovorans* was more effective: in greenhouse experiments using maize seeds treated with this combination at 10^8 cfu/ml, rhizoplane and endorhizosphere

samples were free from colonization by *F. verticillioides*, while rhizoplane and endorhizosphere samples from untreated controls had low levels of colonization.

Other studies indicate that biocontrol bacteria derived from maize are potentially promising alternatives to chemical control strategies. In our laboratory, surveys of maize soil and rhizosphere bacteria for antagonism against *F. verticillioides* were performed (Palumbo et al., 2007), with the goal of isolating bacterial strains for simultaneous control of *F. verticillioides* and *A. flavus*. In liquid co-culture assays using maize kernel extract medium, several strains of *Bacillus*, *Burkholderia*, and *Pseudomonas* showed comparable inhibitory activity against both fungi. From the maize rhizosphere, Hernández-Rodríguez and colleagues (2008) isolated maize rhizosphere strains of *B. cepacia* and *P. fluorescens* that reduced *F. verticillioides* growth on agar by 38% to 68%. In addition, these strains significantly increased plant growth following maize seed treatment, protected maize plants from growth inhibition caused by *F. verticillioides*, and reduced disease incidence by 60% to 86%.

Effects of biocontrol agents on the maize micro-environment inhabited by *F. verticillioides* have also been considered. The impact of seed treatment with individual strains of *B. amyloliquefaciens* and *M. oleovorans* was analyzed relative to indigenous fungal and bacterial populations in the maize rhizosphere (Pereira et al., 2007). Both bacterial treatments effectively reduced *F. verticillioides* populations and fumonisin B1 levels in maize grain samples recovered from plants grown from treated seed. While *F. verticillioides* populations were not recovered from rhizoplane samples of *B. amyloliquefaciens*-treated maize, neither bacterial treatment significantly affected rhizosphere microbial communities, as determined by microbial richness and diversity indices for bacterial and fungal populations. This research suggests that using certain bacterial treatments may selectively target unwanted phytopathogens and mycotoxin producers while preserving native innocuous and beneficial microorganisms in the treated system.

Meanwhile, microbial interactions, and thus biocontrol activities, are also influenced by metabolites produced by *F. verticillioides*. Bacon and colleagues (2004, 2006) showed that fusaric acid (5-butylpicolinic acid), a metabolite produced by all *F. verticillioides*, inhibits growth of *Bacillus mojavensis*, a bacterial

endophyte of maize with biocontrol activity. In vitro experiments showed that fusaric acid was bactericidal to *B. mojavensis*, and that fusaric acid-nonproducing mutant strains of *F. verticillioides* had lower bactericidal effects (Bacon et al., 2004). Exogenous fusaric acid also reduced the production of bacterial inhibitors of *F. verticillioides* growth and reduced endophytic colonization by the bacterium. A survey of *B. mojavensis* isolates indicated that fusaric acid added to media at 100 μg/ml inhibited growth of all strains by 19% to 92%, indicating a natural variation in fusaric acid sensitivity (Bacon et al., 2006). The interaction between fungus and bacterium predicted by these studies reveals one limitation of *B. mojavensis* in biocontrol applications against *F. verticillioides*.

Microbial Interactions with Trichothecene-Producing *Fusarium*

F. graminearum (teleomorph = *Gibberella zeae*), *F. culmorum*, and other *Fusarium* species cause Fusarium head blight and Fusarium seedling blight, depending on the site of infection and the stage of plant development, in wheat and other cereal crops. Trichothecene mycotoxins, including deoxynivalenol (DON, vomitoxin), contribute to pathogenicity in both diseases (Langevin et al., 2004; Wang et al., 2006) and are potential contaminants of harvested and stored grain. Like fumonisin-producing *Fusarium*, the majority of microbial interactions with these trichothecene-producing *Fusarium* species have focused on biological disease control.

Competitive fungi have been investigated as a means of biocontrol of seedling blight in wheat and maize (Luongo et al., 2005). Since crop debris is one major inoculum source for *Fusarium* seedling blight, potentially antagonistic fungi were isolated from cereal crop sources such as stubble, straw, and necrotic plant tissues. Antagonism was determined by measuring the effects of these fungi on *F. culmorum*, *F. graminearum*, *F. proliferatum*, or *F. verticillioides* conidia production in vitro on irradiated wheat straw and maize stubble following co-inoculation. Subsequent bioassays of antagonistic fungal strains were performed under field conditions on maize stalk debris and ears of growing maize co-inoculated with *F. graminearum*, *F. proliferatum*, or *F. verticillioides*. Several isolates of *Clonostachys rosea*, *F. equiseti*, *Chaetomium globosum*, and *Epicoccum nigrum* reduced *Fusarium* populations by

>80% on wheat straw, but generally were less inhibitory on maize stubble.

One *C. rosea* isolate consistently inhibited sporulation of all *Fusarium* species on both substrates. Under field conditions, the percentage of maize stalks colonized by *Fusarium* was significantly reduced by *C. rosea* at one of two field sites. *C. rosea* also inhibited colonization of maize kernels on ears co-inoculated with *F. verticillioides* or *F. proliferatum* by up to 50%. Maize ears were not effectively colonized by *F. graminearum*, suggesting that control of this species would be more likely to be effective on crop debris between growing seasons, rather than during crop production. These experiments suggest that under certain circumstances, naturally occurring fungal antagonists may contribute to the control of *Fusarium* species in agricultural environments. It is tempting to hypothesize that increasing populations of effective fungal species could increase the level of control, although this has not been experimentally tested.

Khan and colleagues (Kahn et al., 2001) isolated antagonistic bacteria and yeasts from wheat anthers and screened them for their capacity to utilize tartaric acid as a carbon source. Tartaric acid is poorly utilized by *F. graminearum*, and so formulations containing tartaric acid–utilizing organisms may have a competitive advantage for carbon sources in situ. Greenhouse screening by point or mist co-inoculations with microbial isolates and *F. graminearum* spores identified *Bacillus* and *Cryptococcus* strains that reduced head blight disease on hard red spring wheat. In point inoculation assays, several isolates significantly decreased disease severity and disease incidence, and significantly increased grain yield. For example, the most effective *Bacillus* isolates reduced disease severity by 77% to 93%, and the most effective *Cryptococcus* isolate reduced disease severity by 56%. In spray inoculation experiments testing the timing of antagonist application, all *Bacillus* and *Cryptococcus* isolates reduced *Fusarium* head blight severity whether they were applied 4 hours before, immediately before, immediately after, or 4 hours after application of *F. graminearum* spores. Subsequent field trials on a number of hard red spring and soft red winter wheat cultivars (Kahn et al., 2004) showed that several of the *Cryptococcus* isolates reduced disease severity by 50% to 60%. DON levels in diseased grain, however, were

not reduced by these antagonists, indicating that in this instance disease suppression and DON production were independent phenomena.

These antagonists were further studied in greenhouse and field trials on durum wheat (Schisler et al., 2002), using spray inoculations of *Bacillus* and *Cryptococcus* strains. Notably, *Bacillus* isolates were more effective in reducing *Fusarium* head blight in greenhouse experiments, while *Cryptococcus* isolates were generally more effective in field trials. For example, in greenhouse assays on the same durum wheat cultivar, one *Bacillus* isolate reduced disease incidence by 78% and disease severity by 92%, while one *Cryptococcus* isolate reduced disease incidence by 10% and disease severity by 43%. In contrast, in field studies at two locations, *Cryptococcus* reduced disease incidence and severity by up to 45% and 57%, respectively, while *Bacillus* reduced disease incidence and severity by up to 31% and 42%, respectively. Grain yield of both greenhouse and field treatments was quite variable, indicating that other ecological or physiological factors were involved. Although the biocontrol efficacy of these bacteria and yeasts was modest, these studies showed the complexity of their interaction with *F. graminearum* under field conditions.

In another study, Palazzini and colleagues (2007) isolated bacteria from wheat anthers and screened them for antagonism without preselection. Fungal inhibition was performed on wheat agar media at different a_w levels and scored by index of dominance. The strains that showed the greatest inhibition of *F. graminearum* growth were *Bacillus* and *Streptomyces* spp. On irradiated wheat grain co-inoculated with *F. graminearum* and bacteria, all of these strains reduced or eliminated measurable DON production. In greenhouse experiments in which bacteria and *F. graminearum* were co-inoculated on individual anthers, none of the bacterial strains reduced disease incidence, but all reduced disease severity. In comparing DON content of spikes from co-inoculated plants, nearly all bacterial treatments significantly reduced DON production, and several strains eliminated measurable DON production. Interestingly, the most effective strains in disease reduction were not the same strains that were the most effective in DON reduction. It remains to be seen whether combinations of effective strains would function in both capacities.

Similarly, another greenhouse study showed that reduced disease severity did not necessarily correlate with decreased DON production (Riungu et al., 2008). Fungal antagonists *Alternaria*, *Epicoccum*, and *Trichoderma* spp. were isolated from wheat kernels and showed mean disease severity reductions of 2%, 7%, and 23%, respectively. Yet while *Alternaria* co-inoculation with *F. graminearum* reduced DON content of harvested wheat grain by 91%, *Trichoderma* treatments increased DON content by over 100%. These data may be the result of *F. graminearum*-fungal interactions in which competitive factors, including DON production, play an as yet unknown role.

In a more recent study by Khan and colleagues (Kahn et al., 2006), bacterial and fungal strains from plant and soil samples were isolated and screened for potential biocontrol efficacy in in vitro seedling germination assays. Wheat seeds inoculated with bacterial or fungal strains were overlaid on potato dextrose agar plates with *F. culmorum* and evaluated for germination after 24 hours incubation. Fifteen bacterial strains, but no fungal strains, reduced seedling germination inhibition by *F. culmorum*. Of these, two *P. fluorescens*, one *P. frederiksbergensis*, one *Pseudomonas* sp., and one *Chryseobacterium* sp. strain significantly reduced disease in stem inoculation assays of wheat and barley. In greenhouse assays, soil amended with strains of *P. fluorescens* or *Pseudomonas* sp. resulted in significant reduction of disease development on wheat and barley following stem base inoculation with *F. culmorum*. Surprisingly, these strains did not show in vitro inhibition of *F. culmorum*, *F. graminearum*, or *F. poae* on agar dual inoculation tests.

In this study, the expression of the trichothecene biosynthetic gene *Tri5*, encoding trichodiene synthase, was inhibited in *F. culmorum* on wheat by the *Pseudomonas* sp. strain by 33% relative to that in wheat inoculated only with *F. culmorum*. Whether this reduction is a direct effect of the fungal-bacterial interaction or a secondary effect resulting from fungal growth inhibition is unknown. Expression of a class III peroxidase, potentially involved in host defense, was induced in wheat co-inoculated with *F. culmorum* and the *Pseudomonas* sp. strain relative to wheat inoculated only with *F. culmorum*. This suggests that one potential mechanism of biocontrol by the bacterium is the induction of plant defenses, though whether this occurs under field conditions remains to be tested.

Lysobacter enzymogenes has also been known to induce systemic resistance in the host plant, has previously been characterized for control of several fungal diseases, and has demonstrated a combination of antibiotic and lytic enzyme production. In testing this bacterium for control of *F. graminearum*, Jochum and colleagues (2006) showed that *L. enzymogenes* significantly reduced disease in several susceptible and resistant wheat cultivars in the greenhouse. However, field trial results were inconsistent from year to year, and were generally less effective than chemical fungicide treatment. The Khan and colleagues (2006) and Jochum and colleagues (2006) studies both demonstrate that in certain cases the ecological interaction of bacteria and *Fusarium* may be largely indirect, via plant responses, rather than directly antagonistic.

A more direct antagonistic interaction within the plant environment was demonstrated by Bacon and Hinton (2007), involving an endophytic *Bacillus mojavensis* strain in wheat. This bacterium produced diffusible compounds that inhibited the growth of 24 strains representing 10 *Fusarium* species associated with seedling blight, regardless of their production of DON. Seed treatment with *B. mojavensis* increased wheat seedling emergence from *Fusarium*-infested soil by 62% in susceptible cultivars and by 26% in a resistant cultivar. Further, colonization of several moderately susceptible to highly susceptible wheat cultivars by *B. mojavensis* restored seed germination and shoot growth in *Fusarium*-infested soil to levels in noninfested soil. This study indicates the potential for *B. mojavensis* and similar endophytic antagonists as agents for biocontrol of wheat seedling blight. Further analyses of *in planta* bacterial-fungal interactions, as well as the effect of *B. mojavensis* on DON production, will provide greater understanding of this phenomenon.

Competitive fungi may also impact trichothecene biosynthesis in pathogenic *Fusarium* species. Using agar diffusion assays, Cooney and colleagues (2001) showed that DON production by *F. graminearum* was significantly inhibited by *Trichoderma harzianum* strains that produced the antibiotic 6-pentyl-α-pyrone (6PAP). These fungi also have the potential to chemically influence each other's secondary metabolism, as shown by increased 6PAP production by *T. harzianum* presumably caused by unidentified diffusible *F. graminearum* metabolites, as well as by reduced DON inhibition in *F. graminearum* strains capable of metabolizing 6PAP.

Other fungi, such as *F. subglutinans*, *F. poae*, *F. equisiti*, and *F. sambucinum*, also showed differing levels of DON inhibition. In competition with fungi that produced different trichothecenes, such as nivalenol-producing strains of *F. culmorum* and *F. crookwellense*, both DON and nivalenol were produced. This study demonstrates one mechanism by which ecological competitors may limit trichothecene production in *Fusarium*, and may lead to insights into the establishment or augmentation of agricultural environments that are suppressive to mycotoxin-producing fungi.

On the other hand, biological control activity may be negatively influenced by the target organisms themselves. Production of DON by *F. culmorum* and *F. graminearum* reduced the expression of the chitinase gene *nag1* in *Trichoderma atroviride*, both on malt extract agar and on maize leaf and stem pieces (Lutz et al., 2003). Repression of gene expression was confirmed using media amended with synthetic DON. A different chitinase gene, *ech42*, was not affected by DON, but synergistic effects of multiple chitinases during mycoparasitic activity of *Trichoderma* may be reduced by interactions with DON. This finding indicates that in addition to environmental factors, the effectiveness of antagonistic microorganisms may be affected by the chemical ecology of the target organism, even at the level of gene expression.

Fungal Interactions with Ochratoxin-Producing *Aspergillus* and *Penicillium*

Ochratoxin A (OTA) is produced by several species of *Aspergillus*, including *A. ochraceus*, *A. carbonarius*, and *A. niger*, as well as *Penicillium verrucosum* and *P. nordicum*. Through these filamentous fungi, OTA contaminates a wide range of agriculturally important crops, including maize, cereals, grapes, and coffee beans. In recent years, microbial interaction studies have largely focused on competition between ochratoxigenic fungi and naturally occurring fungal competitors.

A series of studies by Lee and Magan (1999a, 1999b, 2000) investigated competitiveness of *A. ochraceus* in co-culture with *A. candidus*, *A. flavus*, *A. niger*, *Eurotium amstelodami*, *E. rubrum*, and *Alternaria alternata*, which co-occur as maize spoilage fungi. Carbon utilization patterns determined using Biolog plates as well as 18 predominant maize carbon sources showed differing

niche overlap indices between *A. ochraceus* and the other fungi (Lee and Magan, 1999a). Different a_w levels and different growth temperatures affected the extent of niche overlap. Water stress resulted in lower niche overlap for carbon source utilization between *A. ochraceus* and other *Aspergillus* and *Eurotium* spp., suggesting that *A. ochraceus* may be more competitive under certain environmental conditions. In another study, index of dominance between fungi was examined at three a_w levels and at three temperatures (Lee and Magan, 1999b). On maize meal extract media, *A. ochraceus* was generally dominant in interactions with *A. candidus* and *Eurotium* spp., and dominant to *A. alternata*, *A. flavus*, and *A. niger* under lower a_w conditions.

Comparisons of *A. ochraceus* growth rate and OTA production during fungal co-culture showed that growth rate was generally slower for *A. ochraceus* in co-culture, regardless of the environmental conditions. OTA production was stimulated by co-culture with *A. alternata*, *A. flavus*, and *Eurotium* spp. under particular a_w and temperature conditions. At 30°C and 0.995 a_w, however, *A. candidus*, *A. flavus*, and *A. niger* reduced OTA production. Similar experiments on irradiated maize grain (Lee and Magan, 2000) showed that *A. ochraceus* growth rate was slower in co-culture with other fungi, particularly at 30°C. At higher a_w, *A. ochraceus* was dominant over *A. alternata* and *A. candidus*, but generally not competitive against any fungi at lower a_w. OTA production was not stimulated by any of the fungi on maize grain, but was inhibited by *E. amstelodami*, *A. candidus*, *A. niger*, and *A. flavus*, depending on a_w and temperature. In general, these studies found *A. ochraceus* growth rates were reduced when grown in competition with other filamentous fungi, and OTA production varied according to fungal competitor and environmental condition. These measurements of competition between *A. ochraceus* and other fungi may provide clues as to their in situ behavior, particularly in the case of interactions between ochratoxigenic and aflatoxigenic species, with regard to ecological implications of mycotoxin production.

Competitive fungi were also studied for their interaction with ochratoxigenic *P. verrucosum* in barley (Ramakrishna et al., 1996). Short-term (48 h) co-culture of *P. verrucosum* on barley with *A. flavus*, *F. sporotrichioides*, or *H. burtonii* showed that *H. burtonii* restricted *P. verrucosum* growth at all a_w and temperatures tested, while the other fungal strains had generally no effect.

After 21 days of co-culture on barley grain, *H. burtonii* and *A. flavus* significantly reduced seed infection and *P. verrucosum* populations. Like the Lee and Magan studies, *A. flavus* reduced OTA production at higher a_w and 30°C. OTA was reduced by *H. burtonii* as well, but at lower a_w and 20°C, and to a lesser extent. As with other mycotoxigenic fungi, interaction between *P. verrucosum* and selected fungal competitors can significantly affect both severity of infection and mycotoxigenic potential.

Yeasts have also been studied for their interaction with ochratoxigenic Aspergilli, in grape and coffee systems (Masoud et al., 2005; Bleve et al., 2006). Grape epiphytic yeasts, identified as *Metschnikowia pulcherrima*, *Issatchenkia orientalis*, *I. terricola*, *Kluyveromyces thermotolerans*, and *Candida incommunis* were tested for in vitro inhibitory activity against *A. carbonarius* and *A. niger* strains, representing the major sources of OTA contamination of grape (Bleve et al., 2006). In agar co-culture, *Aspergillus* growth was inhibited by *M. pulcherrima* isolates by 71% to 100%, by *I. orientalis* isolates by 83% to 100%, by *I. terricola* by 74% to 100%, by *K. thermotolerans* by 80% to 100%, and by *C. incommunis* by 74% to 100%. Inhibition was demonstrated to be the result of diffusible antifungal metabolites produced by the yeast strains. On wounded grape berries, *Aspergillus* populations were significantly reduced by co-inoculation with strains of all yeast species other than *K. thermotolerans*, and were most sensitive to *I. orientalis* and *M. pulcherrima* strains, indicating in situ production of antifungal activity.

Epiphytic yeasts were also utilized in controlling ochratoxigenic *A. ochraceus* growing on coffee fruits. Working with volatile organic compounds (VOCs) released by the epiphytic yeasts *Pichia anomala*, *P. kluyveri*, and *Hanseniaspora uvarum*, Masoud and colleagues (2005) showed that yeast VOCs inhibited growth and OTA accumulation of *A. ochraceus* on malt extract agar and on green coffee bean agar. Growth inhibition levels ranged from 25% to 70% on malt extract agar, and 20% to 55% on coffee agar, indicating strain variations in types and amounts of VOCs and medium-dependent sensitivity of *A. ochraceus* to these compounds. OTA accumulation was reduced to undetectable levels by VOCs produced by all yeast isolates under both growth conditions, except for on coffee agar exposed to *H. uvarum* VOCs. The VOCs produced by these yeasts that were most effective in growth

and OTA inhibition were 2-phenyl ethyl acetate, phenyl ethyl alcohol, isobutyl alcohol, isobutyl acetate, and isoamyl alcohol. It was concluded that OTA inhibition was predominantly the result of fungal growth inhibition, rather than from direct exposure to VOCs, as shown by the results obtained with *H. uvarium* on coffee agar. Thus, in addition to diffusible antifungal metabolites produced by environmental competitors, mycotoxigenic fungi may be further influenced by volatile compounds that they produce. Both yeast studies provide insight into microbial interactions occurring on plant microenvironments and demonstrate the potential of naturally occurring microflora as a useful source of biocontrol organisms.

Concluding Remarks

The studies summarized here are indicative of the breadth of microbe-microbe interactions that may naturally occur with mycotoxigenic fungi. The specific interactions examined in each study, typically between one antagonist and one mycotoxin producer, are essential to the dissection of the ecology of these organisms. In order for these interactions to lead to effective biocontrol applications, however, a greater understanding of the long-term, seasonal microbial ecology of the target fungi as well as their potential competitors is needed. Several of these studies showed significant interactions between mycotoxin-producing fungi and bacterial or fungal antagonists in laboratory culture or in greenhouse experiments using sterilized soil. In many cases, these effects were not as apparent in field experiments, where variations in environmental conditions, such as temperature, rainfall, and relative humidity, may play a larger role. In addition, valuable information would be gained by examining how phenotypes important in biocontrol activity affect the interaction between antagonistic test strains and nontarget organisms, including resident populations of similar and dissimilar antagonists. Nevertheless, these recent advances offer useful insights into the ecological diversity of potential antagonistic interactions, which might be exploited for the development of effective biocontrol agents. In addition, microbial interactions resulting in altered mycotoxin content in host crops may provide more clues regarding the ecological basis for mycotoxin production.

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